Nasal immunization with attenuated *Salmonella* Typhimurium expressing an MrpA–TetC fusion protein significantly reduces *Proteus mirabilis* colonization in the mouse urinary tract

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The development of effective strategies to prevent urinary tract infections (UTIs) has become an important goal in public health. *Proteus mirabilis* is commonly associated with complicated UTIs and expresses several virulence factors, including mannose-resistant *Proteus*-like (MR/P) fimbriae. Here, a fusion protein formed from MrpA, the structural protein of MR/P fimbriae, and TetC, a non-toxic but highly immunogenic fragment of tetanus toxin, to be delivered by an attenuated *Salmonella* Typhimurium mutant *in vivo* was constructed. The ability of this strain to induce an immune response and to protect mice against a urinary tract challenge with *P. mirabilis* was investigated. The protein was successfully expressed in *S. Typhimurium*. After two immunization doses, intra-nasally vaccinated mice showed a significant increase in specific serum IgG against MrpA and against *Salmonella* lipopolysaccharide, as well as a significant decrease in kidney and bladder colonization by *P. mirabilis* after challenge. However, no significant correlation was observed between antibody response and kidney or bladder colonization. MrpA fused to TetC and expressed in *S. Typhimurium* effectively protected mice against an experimental *P. mirabilis* UTI.

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INTRODUCTION

Proteus mirabilis is an important cause of a variety of opportunistic and nosocomial infections in humans (Stamm *et al.*, 1977). Although *P. mirabilis* is not the most common cause of urinary tract infections (UTIs) in the normal host (Mobley & Belas, 1995), it can cause severe infections, particularly in patients with urinary catheters or in people with structural abnormalities of the urinary tract (Warren *et al.*, 1982).

UTIs are among the most common infections in humans and represent the most common urological diseases affecting the bladder and kidney (Mobley, 1997). Of all nosocomial infections, 40 % are in the urinary tract and more than 60 % of these are related to an indwelling urinary catheter (Roberts *et al.*, 1990). UTIs also frequently lead to septicaemia in hospitalized patients and about 50 % of those with indwelling catheters for 5 days or more have bacterial colonization of the bladder (Nicolle, 2005).

The design of strategies to prevent and treat UTIs, including the search for effective vaccines, has become a public health concern because of the magnitude of this problem (Nielubowicz & Mobley, 2010). Live attenuated *Salmonella* vaccines have been used successfully as carriers for the delivery of heterologous antigens to the immune system, including those from viruses, bacteria and parasites, eliciting secretory, humoral and cell-mediated immune responses to the recombinant antigens (Chabalgoity *et al.*, 1996; Wang *et al.*, 2010).

Several studies have suggested that mannose-resistant *Proteus*-like (MR/P) fimbriae, which mediate adhesion to uroepithelial cells, may be a candidate vaccine to be used in the prevention of UTIs (Li *et al.*, 2004).

In the present study, a live *Salmonella* vaccine was generated that expressed MrpA, the structural subunit of *P. mirabilis* MR/P fimbriae, as a C-terminal fusion with the TetC protein using the pTECH2 vector (Khan *et al.*, 1994) and its potential value in UTI prevention was assessed using the experimental ascending UTI model in the mouse.

Abbreviations: LPS, lipopolysaccharide; MR/P, mannose-resistant *Proteus*-like; UTI, urinary tract infection.

METHODS

Bacterial strains and plasmids. *Proteus mirabilis* Pr2921 was originally isolated from a patient with symptomatic UTI (Montevideo, Uruguay) (Zunino *et al.*, 2000). *Escherichia coli* XL1-Blue was used for transformation and plasmid stabilization (Bullock *et al.*, 1987). *Salmonella* Typhimurium LB5010, a *galE* mutant, restriction[−] modification⁺ strain (Bullas & Ryu, 1983), was used for transduction of the expression construct using the P22 phage. *S. Typhimurium* BRD509 (SL1344 *aroA aroD*) has been described previously (Roberts *et al.*, 1999). Plasmid pTECH2 (pBR322 ori, *nir* promoter, Ap^R, *tetC*; Khan *et al.*, 1994) was used for cloning of *mrpA*, the gene that encodes the structural fimbrial subunit of *P. mirabilis* MR/P fimbriae, as a fusion protein with TetC.

S. Typhimurium BRD509 will be referred to as BRD509, *S. Typhimurium* pTECH2 as BRD509 pTECH2 and *S. Typhimurium* pTM1 as BRD509 pTM1.

Construct for MrpA expression in the *Salmonella* vaccine strain. *P. mirabilis mrpA* was amplified for cloning into pTECH2 using Pr2921 genomic DNA as a template. Restriction sites were incorporated into the primers, which were designed according to published sequences (Bahrani & Mobley, 1994) (accession number Z32686), excluding the DNA encoding the leader peptide sequence.

Oligonucleotides were as follows: *mrpA* 5' primer, 5'-TCTGCTGGA-TCCGCAGATCAAGGTCATGGT-3'; *mrpA* 3' primer, 5'-CTTATA-CTAGTTTACTGATAAGTCAGTGC-3'. The PCR programme used was: 94 °C for 3 min; 30 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min; and a final cycle of 72 °C for 5 min. The *mrpA* PCR product was gel-purified, subjected to *Bam*HI–*Spe*I digestion and ligated into pTECH2 that had previously been digested with *Bam*HI and *Spe*I. Then, the ligation mixture was used to transform *E. coli* XL1-Blue according to standard procedures (Sambrook *et al.*, 1989).

Transduction of the expression constructs into the *Salmonella* vaccine strain using P22 bacteriophage. The pTECH2-MrpA expression construct (called pTM1) was moved via transformation from *E. coli* XL1-Blue into *S. Typhimurium* LB5010. Then, the transformed strain was grown overnight in Luria–Bertani (LB) broth and 100 µl was added to an equal volume of serial dilutions of a P22 phage stock (10⁷ p.f.u. ml^{−1}) for transduction. This bacteriophage, used for generalized transduction, attaches specifically to the *Salmonella* lipopolysaccharide (LPS) (Susskind & Botstein, 1978). The mixtures were incubated at 37 °C for 20 min to allow phage attachment to the bacteria. Next, 3 ml top agar was added to the tubes and poured onto LB agar plates. Plates were incubated at 37 °C until plaques became visible (4–6 h). Semi-confluent plates were selected and 3 ml SM buffer (Sambrook *et al.*, 1989) was added. After overnight incubation at 4 °C, the buffer was collected, 1% chloroform was added and the tubes were centrifuged at 15 000 r.p.m. for 15 min. The supernatant was filtered (0.22 µm filters; Sartorius) to remove host bacteria. Next, BRD509 was grown overnight in LB broth. The following day, it was diluted 1/50 into LB broth containing 1 mM CaCl₂ and incubated for 2 h at 37 °C. After this, the culture was incubated on ice for 10 min; then, different amounts of the transducing phage stock (1, 5, 10, 50 and 100 µl) were added to culture samples (200 µl). The mixtures were incubated at 37 °C for 1 h and spread onto LB agar/ampicillin (100 µg ml^{−1})/EGTA (5 mM) plates for selection. BRD509 without plasmids was grown on the selection medium as a control. Colonies containing plasmids were also grown on this medium three consecutive times in order to stabilize the plasmid and to prevent the phage entering the lytic cycle by the chelating action of the EGTA. Plasmid DNA was extracted and subjected to restriction enzyme digestion to confirm the presence of the transduced construct.

Expression of MrpA cloned into pTECH2 by *S. Typhimurium*.

The expression assay was performed according to the procedure reported by Khan *et al.* (1994). Selected clones were grown overnight in 5 ml 2 × YT (1.6%, w/v, bacto-tryptone; 1%, w/v, bacto-yeast extract; 0.5%, w/v, NaCl) supplemented with ampicillin (100 µg ml^{−1}) at 37 °C with shaking. A 1/100 dilution in 7 ml 2 × YT/ampicillin (100 µg ml^{−1}) was grown for 3 h to an OD₆₅₀ of 0.4. The tubes were chilled on ice for 10 min and the cells were harvested and resuspended in 300 µl ice-cold PBS-Triton X-100 (0.5%, v/v). Three hundred microlitres 2 × sample buffer was added and the tubes were boiled for 5 min for SDS-PAGE and subsequent Western blot analysis. For Western blotting, an MrpA rabbit polyclonal antiserum previously generated in our laboratory (Scavone *et al.*, 2007) and a rabbit polyclonal anti-TetC antiserum (kindly provided by C. M. Khan, Newcastle University) were used.

Plasmid stability. Once MrpA expression was detected after cloning into pTECH2, the capacity of the construct to be retained in the vaccine strain without antibiotic selection was tested.

To assess this feature, a modification of the procedure reported by Chabalgoity *et al.* (1996) was performed. One colony was grown overnight in LB broth with ampicillin at 37 °C. Next, a 1/1000 dilution of the culture was made in LB broth without ampicillin and grown overnight at 37 °C. Viable plate counts from this culture were made on LB agar without ampicillin and LB agar with ampicillin. Stability (%) was calculated as c.f.u. in LB agar with ampicillin × 100/ c.f.u. in LB agar without ampicillin.

Animals. Three groups of 14 female BALB/c mice were used. All mice used in this study were 8 weeks old. Mice were provided with food pellets and tap water *ad libitum*. All animal experiments were conducted in accordance with procedures authorized by the Bioethics Committee of IIBCE, Montevideo, Uruguay.

Nasal immunization of BALB/c mice with *S. Typhimurium* strains.

Three groups of animals were immunized intranasally with 2 × 10⁹ c.f.u. BRD509 pTECH2, 2 × 10⁹ c.f.u. BRD509 pTM1 or with PBS only (Wang *et al.*, 2010). All bacterial suspensions were in PBS. Immunization was performed by instilling 10 µl bacterial suspension into each nostril (Pellegrino *et al.*, 2003; Scavone *et al.*, 2004, 2009). Vaccination was performed twice on days 1 and 28 (Pickett *et al.*, 2000).

Sample collection. Blood and urine were obtained from each mouse on days 0 and 55 post-immunization for assessment of specific IgG and IgA responses. Urine was obtained by inducing voiding of the bladder by gentle abdominal massage. Blood samples were collected by making a small incision over one of the lateral tail veins. Blood was incubated for 12 h at 4 °C and centrifuged for serum collection. Serum and urine were stored at −20 °C until utilization.

Experimental *P. mirabilis* UTI. Mice were challenged with 2 × 10⁸ c.f.u. *P. mirabilis* Pr2921 suspended in 50 µl PBS using an ascending UTI model (Zunino *et al.*, 2000) on day 56 post-immunization (Pickett *et al.*, 2000). All animals were examined daily and, 7 days after challenge, were killed by cervical dislocation. Bladders and kidneys were removed aseptically and homogenized separately in 10 ml PBS using a Stomacher 80 Lab Blender (Seward). Homogenized tissues were cultured quantitatively on nutrient agar at 2% to inhibit swarming. The numbers of c.f.u. per bladder or kidney were determined after 24 h incubation at 37 °C.

ELISA of specific antibodies in serum and urine against MrpA and LPS. Specific IgG and IgA serum and urine antibodies in individual mice were determined by indirect ELISA (Pellegrino *et al.*, 2003). The plates were coated with 5 µg MrpA ml^{−1} suspended in

carbonate buffer (pH 9.6) per well, followed by incubation overnight at room temperature. For LPS determination, plates were coated with LPS from *S. Typhimurium* ($10 \mu\text{g ml}^{-1}$ per well; Sigma) suspended in carbonate buffer (pH 9.6) and incubated overnight at room temperature. Next, $100 \mu\text{l}$ of a 1:2 dilution of urine and a 1:100 dilution of serum were incubated for 90 min at 37°C for determination of levels of MrpA-specific antibodies. In the case of plates coated with LPS, only 1:100 serum dilutions were performed. For all cases, urine and serum samples of pre-immunized and immunized individual mice were used in duplicate. Bound antibodies were detected by the addition of alkaline phosphatase-labelled goat antibodies specific for mouse IgG (whole molecule) or IgA (α -chain) (Sigma) diluted 1:30 000 or 1:40 000 for IgG and IgA, respectively; plates were incubated for 90 min at 37°C . After washing, *p*-nitrophenyl phosphate (Sigma) substrate (1 mg ml^{-1}) dissolved in diethanolamine buffer was added and plates were incubated for 45 min at room temperature. Finally, A_{405} was measured with an ELISA microplate reader (VarioskanFlash, Skanlt Software 2.4.3 RE). Results were calculated as the difference between A_{405} post-immunization and A_{405} pre-immunization of individual mice in each group (Pellegrino *et al.*, 2003). Data are expressed as means $\pm$ standard deviation for each group of animals.

Statistical analyses. To compare the degree of bacterial colonization in kidneys and bladders, the non-parametric two-tailed Mann-Whitney *U*-test was used. IgG and IgA responses after immunization were analysed by the Duncan test. The correlation between antibody response and bacterial colonization in kidney and bladder for each mouse was measured by Spearman's rank correlation coefficient. Results of all statistical analyses were considered significant when *P* values were <0.05 .

RESULTS

Expression of MrpA by *S. Typhimurium*

MrpA protein was expressed as a C-terminal fusion with the TetC protein (using the pTM1 construct). Expression was detected using a rabbit polyclonal anti-MrpA antiserum (Fig. 1). BRD509 alone and carrying pTECH2 without insert did not show any reactive bands. The clone carrying the MrpA expression construct in pTECH2 showed the predicted 67 kDa band corresponding to the fusion protein and was named BRD509 pTM1. *P. mirabilis* Pr2921 and recombinant MrpA were included as positive controls. Expression of TetC was also evaluated in BRD509 pTM1 using a rabbit polyclonal anti-TetC antiserum. Results are shown in Fig. 1(b). BRD509 without plasmid (negative control) did not exhibit reactive bands. BRD509 pTECH2 without insert showed a 50 kDa band that corresponded to the expressed TetC protein. BRD509 pTM1 showed the predicted 67 kDa band corresponding to the fused protein. Various bands of a lower size, probably products of proteolysis, also reacted with both antisera.

Plasmid stability

The stabilities of pTM1 and pTECH2 in *S. Typhimurium* BRD509 were 96.0 and 90.9 %, respectively. These percentages were considered appropriate for the immunization assay.

Fig. 1. Western blot analysis of *S. Typhimurium* BRD509 vaccine strain expressing MrpA-TetC. (a) Analysis using anti-MrpA polyclonal rabbit antiserum. Lanes: 1, protein marker; 2, recombinant MrpA protein; 3, *P. mirabilis* Pr2921; 4, BRD509; 5, BRD509 pTECH2; 6, BRD509 pTM1. Arrows indicate bands corresponding to expression of MrpA (17.5 kDa) and to expression of the fusion protein MrpA-TetC (67 kDa). (b) Analysis using anti-TetC polyclonal rabbit antiserum. Lanes: 1, protein marker; 2, BRD509; 3, BRD509 pTECH2; 4, BRD509 pTM1. Arrows indicate bands corresponding to expression of TetC (50 kDa) and MrpA-TetC (67 kDa).

Protection of immunized mice against experimental *P. mirabilis* UTI

As shown in Fig. 2, after challenge, a significant decrease in the number of *P. mirabilis* Pr2921 c.f.u. recovered from kidneys was found in the group of mice immunized with BRD509 pTM1 compared to mice immunized with PBS ($P=0.039$) or BRD509 pTECH2 ($P=0.005$). When Pr2921 counts in kidneys of mice treated with PBS and BRD509 pTECH2 were compared, non-significant differences were observed ($P=0.304$).

When bacteria recovered from bladders were analysed, a significant reduction was found in the number of bacteria

Fig. 2. Quantification of *P. mirabilis* ascending experimental UTI. Distribution of c.f.u. in kidneys (a) and bladder (b) of mice challenged after immunization. Each dot represents the $\log_{10}$ c.f.u. recovered from each organ. The limit of detection corresponded to a value of 2 $\log_{10}$ c.f.u. per organ. Significant *P* values were derived using the Mann-Whitney two-tailed non-parametric test. Horizontal bars represent the medians of the $\log_{10}$ c.f.u. per organ of each group. PBS, Mice treated with PBS; BRD509 pTECH2, mice treated with *S. Typhimurium* BRD509 pTECH2; BRD509 pTM1, mice immunized with *S. Typhimurium* BRD509 pTM1.

in the case of mice immunized with BRD509 pTM1 compared with PBS-treated mice ($P=0.04$).

Antibodies in serum and urine

Specific induction of serum and urine IgG and IgA against MrpA and specific serum IgG against LPS was measured by ELISA (Table 1).

Mice immunized with BRD509 pTM1 showed a significant increase in IgG anti-MrpA in serum compared with mice treated with PBS and with BRD509 pTECH2 ($P=0.00007$ and $P=0.035$, respectively).

When serum IgG anti-*S. Typhimurium* LPS was measured, a significant increase was seen in the groups of mice treated with BRD509 pTECH2 and BRD509 pTM1 compared with PBS-treated mice ($P=0.001$ and $P=0.000598$, respectively). Serum IgA anti-MrpA was not significantly induced after immunization in any case.

Significant specific increases of antibodies were not detected in urine in any sample.

Although BRD509 pTM1 immunization reduced the number of *P. mirabilis* c.f.u. in kidneys and bladders and induced significant increases of specific antibodies in serum, no significant association could be established between these parameters ($P>0.05$).

DISCUSSION

MrpA, the structural protein of *P. mirabilis* MR/P fimbriae, was expressed in an *S. Typhimurium* live attenuated

vaccine vector. This system was used in an attempt to investigate whether a live attenuated vaccine for prevention of *P. mirabilis* UTI in mice was feasible.

Although *P. mirabilis* produces several different fimbrial types, the structural subunit MrpA was chosen for this vaccination assay since it had been previously reported to play a crucial role in ascending UTI caused by *P. mirabilis* (Bahrani & Mobley, 1994).

MrpA was expressed as a fusion to TetC, a non-toxic but immunogenic fragment of tetanus toxin. This approach has been used previously for expression of different heterologous antigens like *Schistosoma mansoni* P28 (Khan *et al.*, 1994). For the construction of this recombinant strain, transduction using P22 phage allowed the mobilization of the constructs into smooth-colony phenotype *S. Typhimurium*. So far, no attenuated *Salmonella*-based vaccines have been used to induce protection against UTI.

After mice were intra-nasally vaccinated with *S. Typhimurium* expressing MrpA and challenged with *P. mirabilis* Pr2921, there was a significant decrease in the number of *P. mirabilis* c.f.u. recovered from the kidneys compared to the number of c.f.u. recovered from mice treated with PBS and BRD509 pTECH2 (live attenuated vaccine vector carrying the plasmid without the insertion of *mrpA*). This result indicates that MrpA expression by *Salmonella* conferred to the mice an effective protection against the *P. mirabilis* challenge. Moreover, a significant decrease was also observed in the numbers of c.f.u. recovered from bladders of vaccinated mice compared to the PBS-treated mice. This had not been achieved when another live vaccination approach based on MrpA

Table 1. Antibodies in serum and urine of mice after intranasal immunization

Antibody levels were measured by ELISA in serum (1 : 100 dilutions) and urine (1 : 2). The values represent the means of the differences between A_{405} post-immunization and pre-immunization for individual mice. Data are means $\pm$ SD.

Group	Anti-MrpA antibodies			Anti-LPS antibodies		
	Serum-specific antibodies			Urine-specific antibodies		
	n*	IgG	P	IgA	P	IgG
PBS	14	0.0024 $\pm$ 0.0263		0.0112 $\pm$ 0.02		0.0038 $\pm$ 0.0367
BRD509	14	0.124 $\pm$ 0.085	0.006†	0.0016 $\pm$ 0.025	0.87†	0.127 $\pm$ 0.105
pTECH2						0.001†
BRD509	14	0.209 $\pm$ 0.155	0.00007†, 0.035‡	0.014 $\pm$ 0.0237	0.69†, 0.605‡	0.137 $\pm$ 0.119
pTM1					0.241†, 0.150‡	0.000589†, 0.771‡

*Number of mice.

†Differences between the means of A_{405} of the groups immunized with *Salmonella* strains compared to the group treated with PBS only were considered significant when P was <0.05 using the Duncan test.

‡Differences between the means of A_{405} of the group immunized with BRD509 pTECH2 compared to the group immunized with BRD509 pTM1 were considered significant when P was <0.05 using the Duncan test.

expression by *Lactococcus lactis* was used (Scavone *et al.*, 2007). In all other immunized groups of animals, the numbers of *P. mirabilis* c.f.u. did not significantly decrease compared to the control group treated with PBS.

This study complements other successful approaches for the control of *P. mirabilis* UTI based on vaccination with MR/P fimbrial subunits using different delivery systems. Different degrees of protection have been achieved using different fimbrial proteins, such as MrpA or the fimbrial adhesin MrpH (Li *et al.*, 2004), and also diverse delivery systems have been analysed (Pellegrino *et al.*, 2003; Scavone *et al.*, 2007).

When serum and urine antibody responses were assessed, a significant increase in anti-MrpA IgG was seen in mice immunized with BRD509 pTM1 compared to mice treated with PBS and mice immunized with BRD509 pTECH2 (control strain that did not express MrpA). Although some degree of *Salmonella* fimbrial antigens cross-reaction could be suspected, as mice immunized with BRD509 pTECH2 have an increase in IgG, there is a clear effect due to MrpA expression in BRD509 pTM1 immunized mice. In previous works, it has been observed that sera from MrpA-immunized mice inhibit bacterial adhesion to erythrocytes (Scavone *et al.*, 2009), a finding that was confirmed in this study when sera of mice immunized with BRD509 pTM1 were assayed (data not shown). This result indicates that, at least *in vitro*, specific anti-MrpA antibodies can bind to *P. mirabilis* and inhibit its adhesion to eukaryotic cells.

In addition, a serum IgG anti-*Salmonella* LPS response was achieved in all groups treated with the different vaccine strains, confirming the recognition of the bacterial vector and the antigen delivered by the host.

Correlation of kidney bacterial counts and specific serum IgG induction was not seen in animals immunized with BRD509 pTM1. As in previous works, no significant individual correlation was found between reduction in bacterial counts and antibody levels (Scavone *et al.*, 2007; Pellegrino *et al.*, 2003). The protective role of serum antibodies against mucosal pathogens is still debatable. Probably, serum IgG serves as a back-up for local protection in different mucosal compartments (Renegar *et al.*, 2004).

In this case, we can propose that not only do antibodies play a role in protection, but also innate mechanisms or cellular responses are involved.

In summary, this novel immunization strategy using a living bacterial vaccine vector offers a new approach for the control of UTI caused by *P. mirabilis*.

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